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# Microbial Population Optimization for Control and Improvement of Dark Hydrogen Fermentation

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Additional information is available at the end of the chapter

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## Abstract

Dark hydrogen fermentation (DHF) is a process that can achieve two simultaneous objectives: the production of bioenergy and reduction of pollution. Complex microbiological communities containing efficient producers of hydrogen usually carry out the process. Ordinarily, control and operation strategies optimized the process by chemical and physical factors that usually provide only short-term solutions and adverse effects on microbial properties. Microbial population optimization methods are designed to overcome these problems using knowledge on microbiological aspects, especially regarding optimizing microbial community structure and property. Optimizing microbial community structure and property should be an explicit aim for the (i) design and operation of reactors for DHF process, (ii) creating conditions that select for the stable and productive growth of desired microbes, and (iii) preventing or limiting growth of organisms that would be reducing hydrogen yields. Microbial population optimization could be managed by biostimulation by adding nutrient species specific for their community, bioaugmentation by adding dominant species or efficient hydrogen-producing bacteria into the system, and online process control for maintaining their community.

**Keywords:** dark fermentation processes, biohydrogen production, sludge population optimization, molecular biological techniques, microbial community structure

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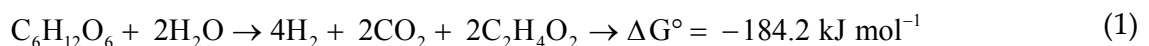
## 1. Introduction

In recent years, the worldwide awareness of global climate change, urban air pollution, and security of future supply of energy carriers stimulates the study on alternative fuels. Hydrogen is a clean and promising fuel when it is ultimately derived from renewable energy sources. It is also efficient and environmentally friendly, as it has high energy content and water is the sole

end product [1, 2]. Today, approximately 95% of commercial hydrogen is generated by steam reforming of natural gas and gasification of coal [3]. As these processes use fossil fuels, they are not environmentally friendly. An alternative way to circumvent the dependence of hydrogen production from fossil fuels is to utilize the potential of hydrogen producing microorganisms to drive hydrogen from widely available biomass. Given these perspectives, biological hydrogen production has high potential as an alternative energy source. Dark fermentative hydrogen production from wastewater yields relatively higher hydrogen production rates than other biohydrogen production processes [4], with the benefit that the substrate cost (wastewater) is free. For example, a fermentative hydrogen-producing process produces hydrogen at a higher rate ( $0.5\text{--}65.0 \text{ l H}_2\text{l}^{-1}\text{d}^{-1}$ ) compared to a light-driven process ( $0.04\text{--}4.3 \text{ l H}_2\text{l}^{-1}\text{d}^{-1}$ ) [5]. In addition, the major advantages are low energy demands, resulting in minimal pollution, operation without light sources, no oxygen limitation problems, and low capital costs for at least small-scale production facilities ( $100\text{--}1000 \text{ m}^3 \text{ H}_2\cdot\text{h}^{-1}$ ) [5, 6–9]. Both mesophilic and thermophilic continuous dark fermentative hydrogen production have been investigated. Thermophilic operation may be particularly appropriate when meeting legislation for the treatment of feedstock containing pathogens or coupled to a process with associated waste heat. Otherwise, because of the energy input needed, thermophilic operation is less likely to be the technically and economically favored option.

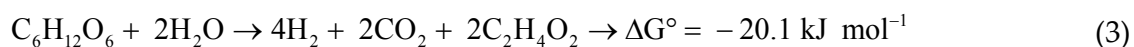
An economically feasible biological approach for hydrogen generation is the conversion of (often negatively valued) organic wastes into hydrogen-rich gas using fermentative bacteria [2, 10]. Various organic waste materials and wastewater from corn, palm oil, soybean, and meat processing plants have been studied for hydrogen production [11, 12]. As dark-fermentative hydrogen production processes involve non-sterile feedstock, mixed microflora derived from natural sources has been commonly used. Theoretically, 4 moles of hydrogen are produced from glucose concomitantly with 2 moles of acetate (Eq. 1,3) with only 2 moles of hydrogen produced when butyrate is the main fermentation product (Eq. 2,4). From the above reactions, it can be concluded that the highest theoretical yield of hydrogen is associated with acetic acid as the fermentation end product. In practice, however, when contents of acetic acid and butyrate in mixture are higher than that of propionate, the yield of hydrogen is higher than in other cases [6, 13]. Typically, 60–70% of the aqueous product during sugar fermentation is butyrate and low hydrogen yields (up to  $2.5\text{--}2.9 \text{ mol H}_2/\text{mol glucose}$ ) compared to the theoretical yield of  $4 \text{ mol H}_2/\text{mol glucose}$  for fermentation with only acetate as liquid end fermentation product [14]. Hydrogen yields can be improved by increasing hydrogen production through reaction (1) and decreasing or preventing reaction (2). This could be accomplished through dark hydrogen fermentation (DHF) with thermophiles or extreme thermophiles, operating at temperatures above  $60^\circ\text{C}$  [15, 16].

Mesophilic ( $35^\circ\text{C}$ )





Thermophilic (60°C)



Higher temperatures thermodynamically favor hydrogen production. Besides, elevated temperatures contribute to better pathogenic destruction and limit hydrogen consumption by hydrogen consumers (methanogens, homoacetogens, sulfate reducers). Normally 67% of the original organic matter will remain in solution (chemical oxygen demand (COD) basis) under optimal conditions of the DHF process. For achieving a full gain of chemical energy preserved in biomass, a coupled process is required that involves the recovery of the remaining organic matter and production of methane, electricity, bioplastics, and hydrogen by photofermentation process. Two-stage processes are already well developed, and they could conceivably be adapted for both hydrogen and methane production [17], and hydrogen and electricity generated from microbial fuel cells [18]. The efficiency of DHF from food waste in anaerobic mesophilic and thermophilic acidogenesis, followed by a two-phase digestion or photofermentation, has also been assessed [19]. Overall, many technologies for the improvement of biohydrogen production have been increasingly examined to determine their likely successful industrial implementation and sustainability for the generation of alternative renewable bioenergy.

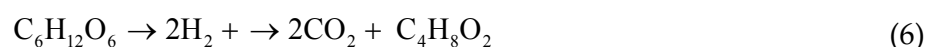
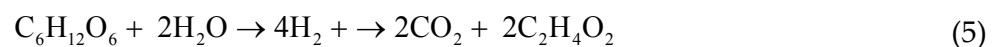
A large number of microbial species, including strict and facultative anaerobic chemoheterotrophs such as Clostridia, Enteric bacteria, *Caldicellulosiruptor* spp., *Thermotoga* spp., and *Thermoanaerobacterium* spp., are efficient producers of hydrogen, while degrading various types of carbohydrates [20]. When using mixed microflora, experimental conditions to suppress methanogenic activity (which consumes hydrogen) and favor hydrogen producing metabolism are necessary. These include inoculum conditioning, optimizing operating conditions such as hydraulic retention time (HRT), pH and substrate concentration, and reducing hydrogen partial pressure [4, 7, 21]. Some challenges for optimizing dark hydrogen fermentation processes have been summarized by Hawkes et al. [7] and there has been considerable progress in research in the last few years, although an economically and technically feasible process is not yet established. In general, control and operation strategies are used to optimize the process by chemical, physical, and biological factors independently that usually provide only short-term solutions by adversely affecting the microbial properties of the system. The process is usually carried out by complex microbiological communities containing efficient producers of hydrogen. Recently, many studies [19, 22–28] have demonstrated molecular evidence related to these various effects. Most of *Clostridium* species have been recognized as desirable bacteria for mesophilic, whereas *Thermoanaerobacterium* species,

*C. thermocellum*, *C. cellulose*, and *C. thermoamyloticum* have been recognized as desirable bacteria for thermophilic conditions. Knowledge and information of microbial community structure and function is the key to improvement of hydrogen productivities through microbial population optimization. Microbial population optimization is a solution based on the existing knowledge of the microbial community data to overcome various technical barriers, such as low hydrogen yields, biomass washout, inhibition by hydrogen, non-stable hydrogen production, and short-time reactor operation. Microbial population optimization requires an integrated knowledge of the microbiology and the physicochemical characteristics of the process. Knowledge on microbiological aspects includes microbial consortia structure and function, the interactions that occur within, and the microbial key players for hydrogen production and their kinetics. The strategies that can be employed following an analysis of the population structure and function include controlling the growth of undesirable microorganisms (i.e., methanogens, propionic acid bacteria, and lactic acid bacteria) that consume hydrogen, while enhancing the numbers and stability of the hydrogen-producing bacteria.

## 2. The dark hydrogen fermentation process

### 2.1. Basic principle for dark hydrogen fermentation

Fermentative hydrogen production yields theoretically a maximum of 4 moles (498 ml-H<sub>2</sub>/g<sup>-1</sup> glucose) of hydrogen from glucose concomitantly with 2 moles of acetate, and 2 moles (249 ml-H<sub>2</sub>/g<sup>-1</sup> glucose) of hydrogen are produced from glucose concomitantly with 1 moles of butyrate. A large number of microbial species, including strict and facultative anaerobic chemoheterotrophs, such as *Clostridia*, enteric bacteria, and *Thermoanaerobacterium*, are efficient producers of hydrogen. Fermentation of glucose to hydrogen, pyruvate, and acetyl CoA, which can be converted to acetyl phosphate, subsequently results in the generation of ATP and the excretion of acetate. Pyruvate oxidation to acetyl CoA requires reduction by ferredoxin (Fd). Reduced Fd is oxidized by hydrogenase, which generates oxidized Fd and releases electrons as molecular hydrogen (Eq.5–8). The practical yield is even lower when other metabolic compounds such as propionate, ethanol, and lactate are produced as the fermentation products. These metabolic products bypass the major hydrogen-producing reaction in carbohydrate fermentation as a consequence of thermodynamic limitations [9].





The proton-reducing ability of  $\text{Fd}_{\text{red}}$  and NADH is thermodynamically limited by the maximum hydrogen partial pressures ( $P_{\text{H}_2}$ ) of 0.3 and  $6 \times 10^{-4}$  atm (60 Pa), respectively. This confers that as long as the  $P_{\text{H}_2}$  is still less than 0.3 atm, hydrogen production can continue with transferring electrons from  $\text{Fd}_{\text{red}}$  which contains electrons from oxidative decarboxylation of pyruvate by pyruvate:ferredoxin oxidoreductase (PFOR). Meanwhile, the oxidation of NADH by NADH:Fd oxidoreductase (NFOR) can generate  $\text{Fd}_{\text{red}}$  that subsequently generates additional hydrogen when the  $P_{\text{H}_2}$  is maintained less than 60 Pa. However, the  $P_{\text{H}_2}$  limited to hydrogen generation via the oxidation of NADH could be increased to 0.1–0.2 atm at a temperature of 70°C [16]. Therefore, increasing cultivation temperature is necessary to overcome thermodynamic limitation, thereby resulting in a decrease of the Gibbs free energy of conversion according to the second law of thermodynamics ( $\Delta G = \Delta H - T \Delta S$ ) [29]. Thermophilic microorganisms produce generally higher hydrogen yields compared to mesophiles because they are thermodynamically favorable [30]. High hydrogen yields in the range of 314.0–473.0 ml- $\text{H}_2/\text{g}^{-1}$  sugars have been previously reported using thermophiles such as *C. thermocellum* and *Thermoanaerobacterium thermosaccharolyticum* and extreme thermophiles such as *Thermotoga elfi*, *Caldicellulosiruptor saccharilyticus*, and *Caldanaerobacter subterraneus* [15, 31–34]. In a practical sense, through controlling the fermentative types of microorganisms, it is possible to maximize the amount of hydrogen produced by fermentation.

## 2.2. Dark hydrogen fermentation by mixed cultures

Dark fermentation process in combination with environmental biotechnology in terms of organic wastes or residue treatment with industrial biotechnology that aims for hydrogen maximization and mixed culture fermentation could thereby become more attractive compared to pure culture fermentation, as mixed cultures are applied originally in the waste treatment fields. Compared to pure culture fermentation, mixed culture fermentation does not require sterilization of the media, offers better adaptation capacity due to its high microbial content and the possibility of mixed substrate co-fermentation, and also allows a continuous fermentation process [35]. Undefined mixed cultures taken from different natural sources need pretreatment or enrichment, by manipulating the operation of the fermentation process and/or by varying the sources of the natural inoculum in order to obtain the required metabolic capacities and the corresponding microbial population for development of the dark fermentation process [36, 37]. To prepare the inoculum for hydrogen production by fermentation of carbohydrates, the original anaerobic sludge is first pretreated to suppress methanogenic archaea, which consume hydrogen generated and subsequently enrich hydrogen-producing bacteria in various reactor configurations [38]. Pretreating anaerobic seed sludge under harsh conditions, spore-forming bacteria involved in anaerobic conversion of carbohydrates to hydrogen could have a better chance to survive compared to the non-spore-forming methanogenic archaea. The spores formed can be activated when the required environmental conditions are provided during subsequent enriching for hydrogen production [39]. Methods,

including heat shock, load shock, acid, base, and chemical pretreatments are usually applied to pretreat anaerobic seed sludge for fermentative hydrogen production

### *2.2.1. Heat shock*

Heat shock has been the most common and effective method for eliminating methanogenic archaea and is achieved by steam heating the seed sludge at 75–121°C with an exposure time between 15 and 120 min, which is relatively easy and inexpensive. The heat shock may also suppress the activity of non-spore-forming propionate producers, but could not effectively deactivate homoacetogens [21, 40]. The existence of homoacetogenic bacteria results in a decrease of hydrogen production because these bacteria further consume hydrogen produced from the fermentation process for the production of acetate [41]. In addition, Duangmanee et al. [42] have previously observed that an inoculum pretreated by heat shock was not stable for hydrogen production in the continuous reactor, and a repeated heat treatment was needed every month to maintain some stability in hydrogen production.

### *2.2.2. Load shock*

During load shock using the pulse load technique in batch and organic fermentation, or hydraulic shock in continuous fermentation, volatile fatty acids (VFAs) tend to accumulate in the fermentative reactor in high concentrations, associated with acidic conditions, and they inhibit methanogens [42, 43]. Applying a load shock with a pulse load of about 40–50 g-sugar/l, the pretreated anaerobic sludge effectively suppressed methanogenic activity [24, 44]. Furthermore, O-Thong et al. [24] have described that load shock-pretreated seed sludge could result in high level of hydrogen production similar to the heat shock-pretreated seed sludge and that load shock would be technically easier to do and more economical than heat shock for implementation on an industrial scale.

### *2.2.3. Acid and alkali pretreatment*

The bioactivity of methanogens during the conventional anaerobic process treatment of organic wastes occurs in neutral to slightly alkaline environments (pH 6.8–8.0) [38]. Limiting methanogenesis can be achieved by adjusting the acidity of the anaerobic sludge substantially away from the preferable range to either pH 3–4 or pH 12. The acid or alkali pretreatment is considered to be technically easier than the heat shock pretreatment for industrial scale implementation [21]; however, the inoculum obtained from an acid or alkali pretreatment requires a much longer acclimatization time of 10 to 30 days to establish hydrogen production [45].

### *2.2.4. Methanogen inhibitors*

2-bromoethanesulfonate acid (BESA), an analog of the coenzyme-M in methanogens, is a chemical that deactivates methanogens. Using BESA at concentrations of 25–100 mM has been found to effectively inhibit the bioactivity of methanogens; however, treating an anaerobic sludge at these levels would not be cost effective for a commercial scale operation [39].

### 3. Molecular methods for microbial community structure and function studies

Molecular monitoring techniques such as fluorescence in situ hybridization (FISH) [46], a combination of FISH and microautoradiography (FISH–MAR) [47], stable isotope probing (SIP) [48], denaturing gradient gel electrophoresis (DGGE) [49], ribosomal intergenic spacer analysis (RISA) [23], and clone libraries have been developed for studying microbial community and function. These methods are used intensively in natural and engineered systems for wastewater treatment. Principles of and deeper insights on these molecular tools are available elsewhere (e.g. [50]). Among these techniques, cloning and the creation of a gene library, DGGE, TRFLP, RISA, and FISH stand out. DGGE was one of the first techniques used to describe DHF microflora [51, 52]. DGGE is a rapid and simple method that provides characteristic band patterns for different samples, allowing quick sample profiling, while retaining the possibility of a more thorough genetic analysis by sequencing of particular bands. DGGE provides information about the structure of microbial communities and can relatively quantify species abundance through DNA band intensities. Cloning provides very precise taxonomical information, but is time consuming and requires specialized personnel and hence, its introduction in the DHF process has been slow. FISH helps identify microorganisms at any desired taxonomical level, depending on the specificity of the probe used. It is the only quantitative molecular biology technique, although quantification is either complex or tedious and subjective. Combination with a confocal laser scanning microscope allows the visualization of three-dimensional microbial structures (granules and biofilms). Both DGGE and FISH have been extensively employed. Other techniques such as RISA [23] provide information on microbial diversity and species dominance. The advantages and disadvantages of the molecular techniques frequently applied to microbial ecology research in DHF process are shown in **Table 1**.

Molecular methods	Nucleic acid extraction	PCR	Advantages	Disadvantages
Fluorescence <i>in situ</i> hybridization (FISH)	No	No	<ul style="list-style-type: none"> <li>- Direct analysis and quantification</li> <li>- Suitable for targeting specific group/species</li> <li>- Easy and fast if required</li> <li>- Allows direct visualization of non-cultured microorganisms</li> <li>- Differential/preferential detection of active microorganisms</li> </ul>	<ul style="list-style-type: none"> <li>- Require genes/RNA with high number of copies</li> <li>- Limit for total diversity mapping</li> <li>- The design of a specific and unambiguously restrictive probe for a certain group of microorganisms is not always possible</li> <li>- The design and optimization of a new probe is a difficult process</li> <li>- Structural analysis of granular requires a confocal microscope</li> </ul>
Denaturing gradient gel	yes	yes	<ul style="list-style-type: none"> <li>- Permits rapid and simple monitoring of the spatial</li> </ul>	<ul style="list-style-type: none"> <li>- Bias from PCR</li> </ul>



Molecular methods	Nucleic acid extraction	PCR	Advantages	Disadvantages
electrophoresis (DGGE)			<ul style="list-style-type: none"> <li>temporal variability of microbial populations if just band patterns are considered</li> <li>- It is relatively easy to obtain an overview of the dominant species of an ecosystem</li> <li>- It is adequate for analysis of a large number of samples (far more than cloning)</li> </ul>	<ul style="list-style-type: none"> <li>- The number of detected bands is usually small, which implies: the number of identified species is also small; the bands correspond, although not necessarily, to the predominant species in the original sample</li> <li>- The sequences of the bands obtained from a gel correspond to short DNA fragments (200–600 bp), and so phylogenetic relations are less reliably established than with cloning of the whole 16S rRNA gene. In addition, short sequences are less useful for designing new specific primers and probes.</li> <li>- GC content of the amplified DNA can modulate the <i>Tag</i> polymerase activity</li> </ul>
Cloning and sequencing	Yes	Yes	<ul style="list-style-type: none"> <li>- Contain larger sequence</li> <li>- Complete 16S rRNA sequencing allows: very precise taxonomic studies and phylogenetic trees of high resolution to be obtained</li> <li>- Identification of microorganisms that have not been yet cultured or identified</li> <li>- Covers most microorganisms, including minority groups, which would be hard to detect with genetic fingerprinting methods</li> </ul>	<ul style="list-style-type: none"> <li>- A large number of clones must be sequenced for positive diversity</li> <li>- Sequences need to be compared with each other and libraries</li> <li>- Very time consuming and laborious, making it unpractical for high sample throughput</li> <li>- It is not quantitative. The PCR step can favor certain species due to differences in DNA target site accessibility</li> <li>- Bias from PCR, total universal primers cannot be totally universal bacteria</li> <li>- Exponential amplification of the DNA mixture may result in ratio discrepancies between the amplified 16S rDNA fragments and the original mixture</li> </ul>

**Table 1.** Brief description of frequently molecular methods that have been used for microbial community analysis in dark fermentative hydrogen production.

For decades, a biological reactor has been considered as a black box. The new insights in microbiology have helped to improve the design and performance of new generation reactors [53, 54]. Probably it is true that it is not essential to know the phylogenetic position of the individual microorganisms that dwell inside a system for the design of a biohydrogen facility. But the knowledge on microbial community structure and function is needed. The more recent reports on microbial community structures of DHF processes still interpret the results with reactor performance and metabolic by-products (indirect function) [55, 56]. However, we are

still uncertain regarding which microorganisms can function effectively in DHF and whether the whole community takes part. Thus, deeper insight into the function is required, not just community structure. The latter is due to a general shortcoming in all these molecular tools. However, some attempts have been made in this direction, as FISH–MAR and FISH combined with biosensors could be applied to reveal the microbial community structure and function in parallel. Furthermore, other techniques such as DNA microarrays are being developed with the goal of being able to infer the in situ physiology of the microorganisms [57], and these should find application in the hydrogen-producing biosystems.

Post-genomic research and systems biology tools such as metaproteomics will greatly contribute to the development by providing functional performance insights of the microorganisms and their metabolism [55]. Recent work on post-genomics involving microbial ecosystems has expanded to both natural microbial biofilms and activated sludge [56, 58]. These cutting-edge technologies are aimed at using new molecular tools to understand the microbial community structures in relation to functions [55] or metabolic transformations [58]. It is commonly known that 16S rRNA genes approaches have copy numbers and PCR bias problems. Housekeeping genes with a single copy are now the focus for population genomic analysis. Multilocus sequence typing (MLST) of housekeeping genes could provide a deeper insight on how microbial populations evolved [59]. These modern molecular monitoring techniques are vital tools and could also be applied for DHF, as they will particularly break new ground for the quantification and dynamics of microorganisms in complex consortia.

A whole variety of analytical methods for both microbial community structure and function are now available. However, microbiologists and engineers should take efforts to apply these tools for quantitative studies of DHF. With a more thorough understanding of the microbial community and its dynamics, an improvement of expectations and optimization of fermentative processes will be possible. The microbial community structure and microbial function may be further optimized by adding species and specific nutrients for the dominant species.

## **4. Molecular evidence in dark hydrogen fermentation processes**

### **4.1. Effect of inoculum types and conditioning on microbial community structure**

It has been previously reported that the methods for seed preparation can affect both start up and overall efficiency of the hydrogen-producing reactors [7]. Quick recovery from process upsets in full-scale applications may also require large quantities of readily available hydrogen-producing seeds. Therefore, induction of hydrogen accumulation in fermentative consortia is related to the inhibition of hydrogen consumers which is essential for its further scale-up and industrial applications. Several types of inocula have been used for anaerobic hydrogen fermentation, such as anaerobic-digested sludge [60], sewage treatment sludge [61], agricultural soil [62], sludge compost [63], and isolated bacteria [64]. In addition, several methods are used for conditioning inocula such as acid conditioning [65], heat conditioning [60, 62], chemicals conditioning such as 2-bromoethanesulfonic acid (BESA) [66], short hydraulic retention time (HRT) without conditioning [67], and overload conditioning [24]. All condi-

tioning methods aid in inhibiting methane formation, as well as accelerating the enrichment of hydrogen producing bacteria, such as spore-forming *Clostridium* species, as these are highly tolerant to extreme environments [68]. The effect of conditioning on hydrogen production rates is inoculum dependent, with appreciable hydrogen production yields being demonstrated with anaerobic-digested sludge and agricultural soil [69]. Several studies (e.g. [23, 51, 70–73]) reveal that heat-conditioned anaerobic-digested sludge guarantees the highest hydrogen production yields. Heat shock treatment of sludge gave highest hydrogen yield (2 mol H<sub>2</sub>/mol glucose), while base treatment of sludge gave lowest hydrogen yield (0.48 mol H<sub>2</sub>/mol glucose) [74]. Sung et al. [71] illustrated that hydrogen production using heat-treated seeds declined after 1-month operation and repeated heat treatment of sludge to recover from reactor every month is not credible. However, others claim that high yields can be achieved without heat treatment [68]. Zhu and Beland [75] have demonstrated that heat shock and acid treatment methods completely repressed methanogenic activity, while base treatment methods did not completely repress methanogenic activity and also significantly affected hydrogen production. Hwang et al. [76] reported that the acidic conditions (pH 4.5–6) can act as a weak inhibitor, but not complete long-term inhibition of methanogenic activity. Elsewhere, it has been shown that acid pretreatment is particularly effective for enhancing the growth of lactic acid bacteria (LAB) [52, 77]. Five methods for preparation of hydrogen-producing seeds (base, acid, 2-bromoethanesulfonic acid (BESA), and load shock and heat shock treatments) as well as an untreated anaerobic digested sludge were evaluated for their hydrogen production performance and responsible microbial community structures under thermophilic conditions (60°C) by O-Thong et al. [24]. The results showed that the load shock treatment method was the best for enriching thermophilic hydrogen-producing seeds from mixed anaerobic cultures as it completely repressed methanogenic activity and gave a maximum hydrogen production yield of 1.96 mol H<sub>2</sub> mol<sup>-1</sup> hexose with a hydrogen production rate of 11.2 mmol H<sub>2</sub> l<sup>-1</sup> h<sup>-1</sup>.

In general, microbial profiles in fermentative production processes occur as a result of a combination of process conditions, such as feedstock characteristics, environmental conditions (pH, temperature, and H<sub>2</sub> partial pressure), and metabolic pathways existing in the microbes involved [51]. Iyer et al. [51] investigated hydrogen-producing bacterial communities from a heat-treated soil inoculum by RISA. They found that species of Clostridiaceae, Bacillaceae, and Enterobacteriaceae responded to hydrogen production at 30°C and a 30-h HRT. The gene pool at 30-h HRT, as determined by 16S rRNA gene sequences, was more diverse than at the 10-h HRT, as only Clostridiaceae were detected at this later point. The application of DGGE indicated *Clostridium tyrobutyricum*, *Lactobacillus ferintoshensis*, *Lactobacillus paracasei*, and *Coprothermobacter* species to be dominant in bacterial communities developed from pH-pretreated inocula [68]. *Lactobacillus* species are common coexisting bacteria in hydrogen fermentation processes. However, they have adverse effects on hydrogen production by competing for sugars and producing acidic products [78, 79]. Interference by lactic acid bacteria is often prevented by feedstock heat treatment at 50°C or by thermophilic fermentation at temperatures beyond 50°C [80]. Load shock and heat shock treatments under thermophilic conditions resulted in a dominance of *T. thermosaccharolyticum* while base- and acid-treated seeds were dominated by *Clostridium* and BESA-treated seeds were dominated by *Bacillus* sp. [24]. The comparative experimental results from hydrogen production performance and

microbial community analysis showed that the load shock treatment method was better than base- and acid-treated, heat shock, BESA-treated methods for enriching thermophilic hydrogen-producing seeds from anaerobic-digested sludge. Load shock-treated sludge was implemented in palm oil mill effluent (POME) fermentation and was found to give maximum hydrogen production rates of 13.34 mmol H<sub>2</sub> l<sup>-1</sup>h<sup>-1</sup> and resulted in a dominance of *Thermoanaerobacterium* spp. Load shock treatment is an easy and practical method for enriching thermophilic hydrogen-producing bacteria from anaerobic-digested sludge. The efficiency of preparation methods could be considered based on hydrogen production yield together with microorganisms revealed in the process. Therefore, the microbiological aspects and hydrogen production performance information are needed to identify effective methods for preparation of hydrogen-producing seeds.

#### 4.2. Effect of reactor design and operation on microbial community structure

Various reactor types seeded with the same inoculum and operating under similar process conditions could develop microbial communities with different properties. For instance, in batch mode under mesophilic conditions with glucose as a substrate, microbial communities became dominated by *Clostridium butyricum*-like species [51], *Clostridium* spp. [52] *C. butyricum* [81], and *Clostridium* sp\_T5zd [77]. Conversely, a continuous stirred tank reactor (CSTR) was dominated by *Clostridium sporogenes*-like and *Clostridium celerecrescens*-like species [82]. Yet, in an anaerobic membrane reactor (MBR), the main population consisted of Clostridiaceae, Flexibacteraceae, *Clostridium acidisoli*, *Limningia china*, and *Cytophaga* [23]. *Clostridium* spp. were also dominant in a CSTR used to produce hydrogen from sucrose at 35°C, pH 5.5, and HRT 12 h, as analyzed by DGGE [51, 71]. Xing et al. [83] followed communities in a CSTR operating on molasses at a low pH with acidophilic bacteria from sewage, which established an ethanol–acetate hydrogen-producing community after 28 days. This was also consistent with other studies, i.e., the hydrogen production rate increased with the increase of *Ethanoligenbacterium* sp., *Clostridium* sp., and *Spirochaetes*. Some types of *Clostridium* sp., *Acidovorax* sp., *Kluyvera* sp., and *Bacteriodes* were found throughout all periods of reactor operation [83]. It appeared that hydrogen production depended not only on hydrogen producers but also on cometabolism in the whole community.

In common with many other systems, in batch fermentations without pH control, it has been found that microbial communities change with pH [77], and their biodiversity decreased considerably as the pH decreased from 6.5 to 4.5. Kim et al. [81] reported the effect of substrate concentration on dark hydrogen fermentation using a CSTR. At the peak of hydrogen production yields, all bacterial species detected by DGGE analysis were *Clostridium* spp. and at inlet sucrose concentrations below 20 gCOD l<sup>-1</sup>, the hydrogen yield per hexose consumed decreased, while *Clostridium scatologenes* (an H<sub>2</sub>-consuming acetogen) was found in the sludge. Moreover, it has been shown that short HRT operation without anaerobic sludge preparation allowed for more microbial diversity and increasing the system robustness [22]. Species that differ in optimal growth conditions but are metabolically similar are then present, sharing the same function. Such advantages allow for flexibility in performance when perturbations in process conditions occur. Overall, under mesophilic conditions, hydrogen may be produced

by a large group of bacteria such as the three main groups belonging to the low-GC(guanine-cytosine)gram positive bacteria, i.e., Clostridiaceae, Enterobacteriaceae, and Bacillaceae. A number of studies have focused on the analysis of the 16S rRNA gene to understand the species richness of microbial communities in lab-scale reactors under mesophilic conditions, as shown in **Table 2**.

Substrate	Processes and operation condition	Dominating microorganisms	H <sub>2</sub> yield (mol H <sub>2</sub> mol <sup>-1</sup> hexose)	H <sub>2</sub> rates (l H <sub>2</sub> l <sup>-1</sup> d <sup>-1</sup> )	References
Glucose	Batch experiment, pH 5.5 and 36°C	Clostridiaceae Enterobacteriaceae <i>Streptococcus bovis</i>	0.47	4.6	[52]
Carbohydrate-containing wastewater	Two-step process using CSTR, pH 5.5, HRT 6 h, 36°C and complete-mix cylindrical photoreactor, HRT 25 h, pH 8.0, 32°C	<i>Clostridia</i> <i>Rhodobacter capsulatus</i>	2.1 and 2.5	4.5 and 0.3	[84]
Glucose	Anaerobic membrane reactor, HRT 3.3 h, pH 5.5, mixed at 200 rpm and 35°C	Clostridiaceae Flexibacteraceae <i>Clostridium acidisoli</i> <i>Linmingia china</i> <i>Cytophaga</i>	1.1	15.36	[23]
Food waste	CSTR, HRT 5 d, pH 5.6 and 35°C	<i>Thermotogales</i> <i>Bacillus</i> spp. <i>Prevotella species</i>	0.03–0.1	0.22	[19]
Sucrose	CSTR, HRT 24 h, 37°C and pH 5.5	<i>Clostridium</i> sp. <i>Bacillus</i> sp.	2.3	0.1	[71]
Glucose	CSTR, pH 5.5 and 30°C at 30 h and 10 h HRT	Bacillaceae Clostridiaceae Enterobacteriaceae Only Clostridiaceae at HRT 10 h	1.61	10.4	[51]
Rice slurry	Batch experiment, pH 4.5 and 45°C	<i>Clostridium</i> sp. 44a-T5zd	2.5	2.1	[77]
Sucrose	CSTR, gas sparging at 300 ml/min, pH 5.3 and 35°C	<i>Clostridium tyrobutyricum</i> <i>Clostridium ptoteolyticum</i> <i>Clostridium acidisoli</i>	1.68	6.45	[81]
Sucrose	CSTR, HRT 12 h, pH 6.8 and 35°C	<i>Clostridium ramosum</i>	0.9–3.5	9.1	[85]
Glucose	CSTR; glucose to peptone ratio (5:3) 35°C, pH 7 and HRT 12 h	<i>Clostridium sporogenes</i> <i>Clostridium celerecrescens</i>	0.6	6.8	[82]
Glucose	Batch; glucose to peptone ratio (5:3) 35°C, pH 7 and HRT 12 h	<i>Clostridium butyricum</i>	1.11	5.2	[83]

**Table 2.** Microbial community structure, operational conditions and reactor performance of fermentative hydrogen production process from various organic wastes under mesophilic condition.

Different microbial community structures develop within different temperature regimes. For instance, a comparative study on hydrogen production from food waste between mesophilic and thermophilic acidogenic conditions revealed that biogas produced in thermophilic conditions was methane free, whereas methane was still detected under mesophilic conditions [19]. Species such as *Thermoanaerobacterium thermosaccharolyticum* and *Desulfotomaculum geothermicum* were detected in the thermophilic acidogenic culture, while *Clostridium* and *Bacillus* species were detected in the mesophilic acidogenic culture with DGGE. The composition of microbial communities in thermophilic dark hydrogen fermentation production was investigated in more detail using quantitative FISH and DGGE [8, 22, 86]. This demonstrated that *Thermoanaerobacterium* made up almost half of the total community in thermophilic dark hydrogen fermentation production.

In thermophilic fermentative hydrogen production, a number of microbial species are known, including *C. thermoamylolyticum* [84], *C. cellulose*, *C. thermocellum*, *T. thermosaccharolyticum* [22, 84], *D. geothermicum* [19], *Saccharococcus* sp. clone ETV-T2 [8], *Mitsuokella jalaludinii* [84]. *Thermoanaerobacterium* sp. and the related genotypes are found to be dominant in many thermophilic fermentations operating at 55°C and neutral pH with feedstocks, including starch, organic waste [22], and cellulose-rich materials [84]. Thus, many studies on microbial consortia of thermophilic fermentations resulted in the detection of the same dominant species. This is in contrast to observations from mesophilic fermentations, and it might therefore indicate that thermophilic conditions lead to a convergence of microbial populations. In this way, thermophilic reactors can provide an additional benefit for the application in sludge population optimization. One of the problems of bioreactor operation is washout of microorganisms. Trickle biofilter reactors (TBR) have been proposed as a solution to this problem, with continuous hydrogen production under thermophilic conditions being successfully demonstrated [22, 23]. In those studies, the TBR was dominated by *T. thermosaccharolyticum* and *Clostridia* and *Bacilli* in the phylum *Firmicutes*.

Microbial community structure dynamics in the ASBR for biohydrogen production from palm oil mill effluent during changing of hydraulic retention time (HRT) and organic loading rate (OLR) was studied by denaturing gradient gel electrophoresis (DGGE) aiming at improved insight into the hydrogen fermentation microorganisms. The microbial community structure was strongly dependent on the HRT and OLR. DGGE profiling illustrated that *Thermoanaerobacterium* spp., such as *T. thermosaccharolyticum*, and *T. bryantii*, were dominant and probably played an important role in hydrogen production under thermophilic conditions. The shift in the microbial community from a dominance of *T. thermosaccharolyticum* to a community where *Caloramator proteoclasticus* also constituted a major component occurred at suboptimal HRT (1 d) and OLR (80 gCOD l<sup>-1</sup>d<sup>-1</sup>) conditions [25]. The information showed that the hydrogen production performance was closely correlated with the bacterial community structure. A number of studies have focused on the analysis of the 16S rRNA gene to understand the species richness of microbial communities in lab-scale reactors under thermophilic conditions, as shown in **Table 3**.

Substrate	Processes and operation condition	Dominating microorganisms	H <sub>2</sub> yield (mol H <sub>2</sub> rates (l hexose)		References
			H <sub>2</sub> mol <sup>-1</sup>	H <sub>2</sub> l <sup>-1</sup> d <sup>-1</sup> )	
Glucose	Fed batch experiment, HRT 0.5 d, pH 6.6, and 60°C	<i>Thermoanaerobacterium thermosaccharolyticum</i> KU-001	2.4	3.5	[70]
Cellulose	Batch experiment, stirring at 200 rpm, pH 6.4, and 60°C	<i>Clostridium</i> and <i>Bacillus</i> <i>T. thermosaccharolyticum</i> <i>Clostridium thermocellum</i> <i>Clostridium cellulosi</i>	2.0	1.35	[87]
Starch in wastewater	Batch experiment, pH 6.0 and 55°C	<i>Thermoanaerobacteriaceae</i> <i>Saccharococcus</i> sp. clone ETV-T2	0.68	2.8	[8]
Cellulose	Batch experiment, pH 6.5 and 55°C	<i>Thermoanaerobacterium</i> <i>Clostridium thermoamylolyticum</i>	0.2	0.82	[84]
Food waste	CSTR, HRT 5 d, pH 5.6, and 55°C	<i>Thermoanaerobacterium thermosaccharolyticum</i> <i>Desulfotomaculum geothermicum</i>	0.9–1.8	4.56	[19]
Glucose	Trickling biofilter reactor (TBR), HRT 2 h and 55–64°C	<i>T. thermosaccharolyticum</i>	1.1	23.25	[22]
Food waste	CSTR; HRT 5 d, pH 5.5, and 55°C	<i>T. thermosaccharolyticum</i>	2.2	1.4	[86]
Artificial garbage slurry	Jar fermentor; HRT 1d, pH 6.0, and 60°C	<i>T. thermosaccharolyticum</i>	1.99	4.46	[88]

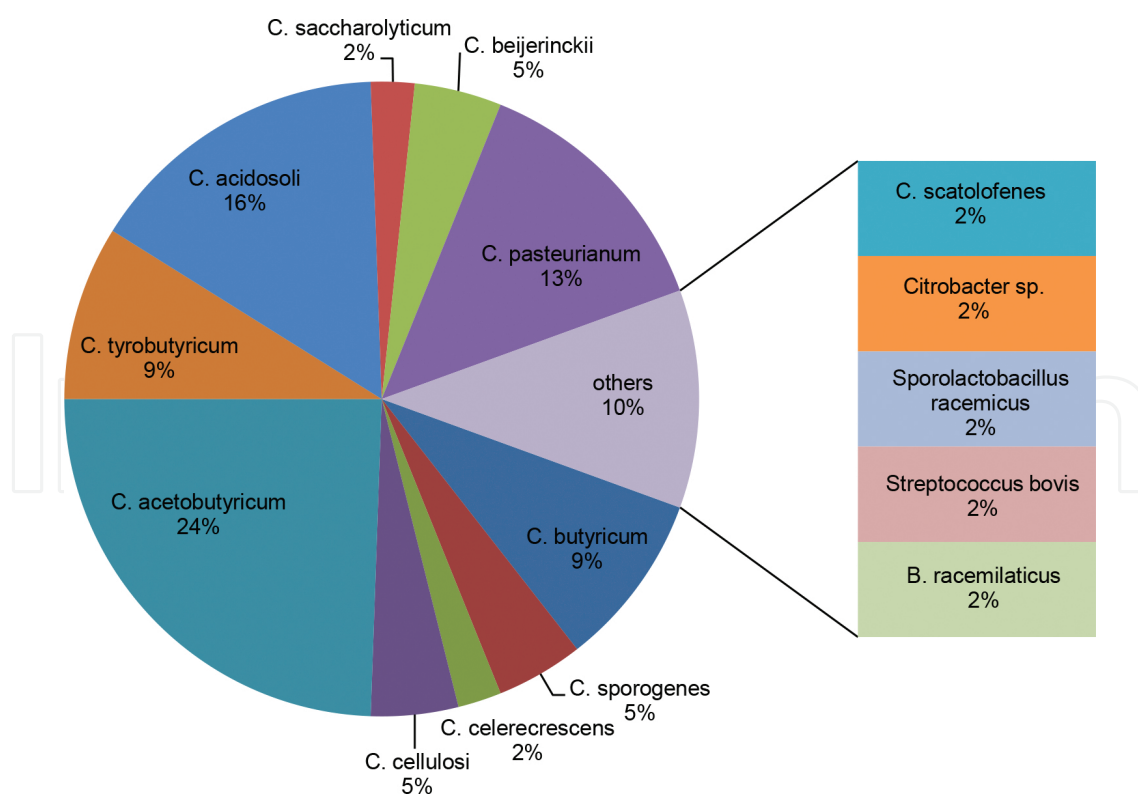
**Table 3.** Microbial community structure, operational conditions and reactor performance of fermentative hydrogen production from various organic wastes under thermophilic condition.

In addition to volatile fatty acids (VFAs), anaerobic fermentations may also lead to the production of reduced end products such as ethanol, butanol, and lactate [5], thus reducing H<sub>2</sub> yield potential. Therefore, bacterial metabolism must avoid VFAs by efficient product removal [7, 8] or metabolic engineering. Stripping gas may be used to remove H<sub>2</sub> from the liquid phase to prevent product inhibition. N<sub>2</sub> is used often, but it increases the costs of H<sub>2</sub> purification. For economical reasons, CO<sub>2</sub> might be a better choice, as it is relatively easy to separate from the gas phase. Using CO<sub>2</sub> rather than N<sub>2</sub> for stripping H<sub>2</sub> resulted in a higher production of H<sub>2</sub> and butyrate [79, 89]. High CO<sub>2</sub> partial pressures had little effect on hydrogen-producing bacteria but were inhibitory to other competitive microorganisms such as acetogens and lactic acid bacteria. The microbial community structure under CO<sub>2</sub> sparging conditions was dominated by *C. tyrobutylicum*, *C. proteolyticum*, and *C. acidisoli*. CO<sub>2</sub> sparging has another

beneficial effect on reactor performance by improving mixing and contact between substrate and microorganisms and also decreased the effects of hydrogen partial pressure [89, 90].

### 4.3. Microbial key players in dark hydrogen fermentation

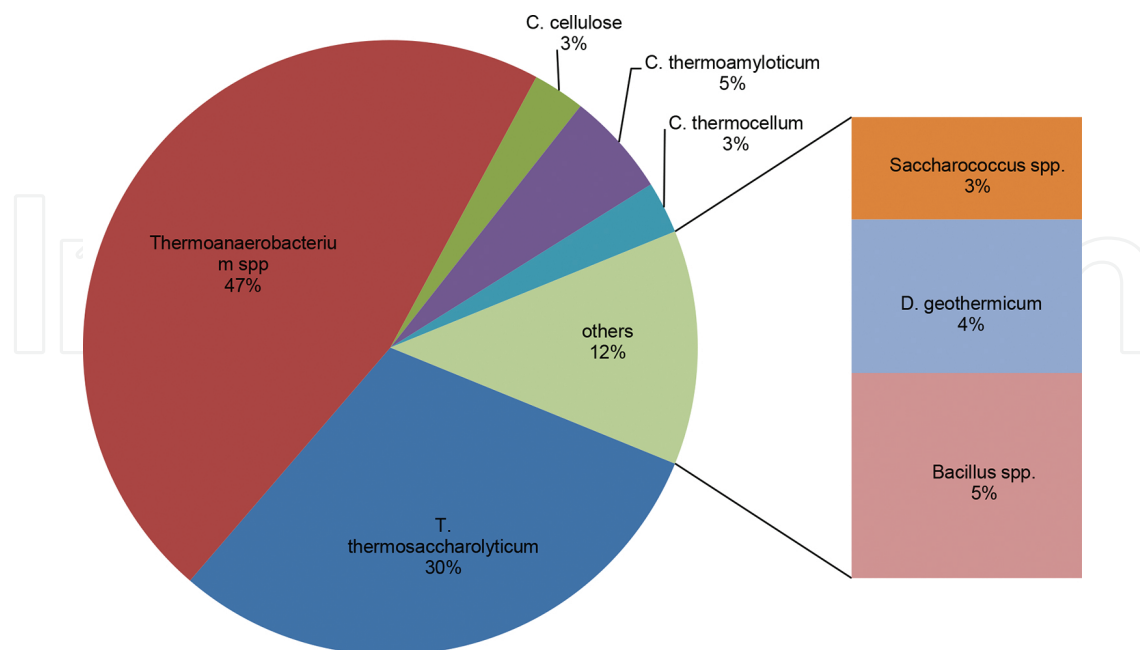
**Figure 1** summarizes the richness of the microbial key players of mesophiles. Fermentative hydrogen production has been studied for a large group of pure cultures, including species of *Enterobacter*, *Bacillus*, and *Clostridium*. However, hydrogen-producing microflora obtained from natural sources, which are able to survive on non-sterile substrates, contain mostly *Clostridium* spp., such as *C. butyricum*, *C. acidisoli*, *C. tyrobutylicum*, and *C. acetobutylicum*. Although the numbers of case studies are still low to infer solid conclusions, they indicate that the *Clostridium* genus represents the major group in dark mesophilic fermentation under mesophilic conditions. Various *Clostridium* species are found in mesophilic environments, but only four species are highly frequently observed (*C. acetobutylicum* (24%), *C. tyrobutyricum* (9%), *C. acidisoli* (16%), and *C. pasteurianum* (13%)) and related with high hydrogen yield [52,81]. However, *C. saccharolyticum*, *C. butyricum*, *C. sporogenes*, *C. celerecrescens*, *C. cellulosi*, and *C. beijerinckii* were also found to be strong hydrogen producers [83]. Others species (*Citrobacter* sp., *Sporolactobacillus racemicus*, *Streptococcus bovis*, and *B. racemilaticus*) that differ in optimal growth conditions from *Clostridium* but are metabolically similar are allow for flexibility in performance when perturbations in process conditions occur.



**Figure 1.** Summary of all fermentative hydrogen-producing bacteria frequently observed based on molecular tools studied under mesophilic conditions.



The *Thermoanaerobacterium* genus represents the major group in dark thermophilic fermentation. **Figure 2** summarizes the richness of the microbial key players of thermophiles. Thermophilic conditions clearly show that *T. thermosaccharolyticum* is a key player in fermentative hydrogen production. *Thermoanaerobacterium* spp. have also been found to dominate in a long-term hydrogen production reactor. Bacteria species are highly frequently observed under thermophilic conditions and they are *Thermoanaerobacterium* sp. (47%) and *T. thermosaccharolyticum* (30%). The microbial community structure of thermophilic mixed culture sludge used for biohydrogen production from palm oil mill effluent was analyzed by fluorescence in situ hybridization (FISH) and 16S rRNA gene clone library techniques. The microbial community was dominated by *Thermoanaerobacterium* species (~66%). The remaining microorganisms belonged to *Clostridium* and *Desulfotomaculum* spp. (~28% and ~6%, respectively). The hydrogen-producing bacteria were isolated and their ability to produce hydrogen was confirmed. Three hydrogen-producing strains, namely HPB-1, HPB-2, and HPB-3, were isolated. The 16S rRNA gene sequence analysis of HPB-1 and HPB-2 revealed a high similarity to *T. thermosaccharolyticum* (98.6% and 99.0%, respectively). The *Thermoanaerobacterium* sp. HPB-2 strain was a promising candidate for thermophilic fermentative hydrogen production with a hydrogen yield of 2.53 mol H<sub>2</sub> mol<sup>-1</sup> hexose from organic waste and wastewater containing a mixture of hexose and pentose sugars. *Thermoanaerobacterium* species play a major role in thermophilic hydrogen production as confirmed by both molecular and cultivation-based analyses [91]. Various *Clostridium* species (*C. cellulose*, *C. thermoamylicum*, and *C. thermocellum*) that differ in optimal growth conditions from *Thermoanaerobacterium* but are metabolically similar are allowed for flexibility in performance when perturbations in process conditions occur. Other species (*Saccharococcus* spp., *D. geothermicum*, and *Bacillus* spp.) could be allowed for more microbial diversity and increasing the system robustness.



**Figure 2.** Summary of all fermentative hydrogen producing bacteria frequently observed based on molecular tools studied under thermophilic conditions.

## 5. Microbial population optimization for dark hydrogen fermentation

Different species likely possess different growth properties (growth rates, affinity constants with substrates, and yields), and perhaps different capacities in coping with stress arising from variations in growth conditions. Obviously, the species with the most desirable properties would be selected to perform a required function. The possibility of selecting species with better properties has huge potential for improving the performance (efficiency and reliability) of a DHF system. Unfortunately, we still lack knowledge concerning the species to be selected and how they may be selected. Furthermore, 16S rRNA sequence-based identification does not allow inference of functional properties. The correlation between microbial community composition and reactor performance would provide a rationale to further improve the efficiency of fermentative hydrogen production. The characterization of the microbial community as a whole contributes to meaningful data regarding structure and function of such communities and their activities.

The interest in hydrogen as a clean energy carrier has strongly increased recently. Cost-effective generation of hydrogen through fermentation will have an important role in making this idea a reality. Future dark hydrogen fermentation from organic wastes depends on a thorough understanding of the microbiological community structure and function for enhanced or controllable hydrogen production and reactor. Sludge population optimization aims to obtain the best performance of a system through maximizing the properties of the sludge such as kinetics, yields, and robustness to environmental disturbance. A systematic investigation on the effects of a number of operational conditions on fermentative hydrogen production community and their properties is essential for sludge population optimization. The operational parameters to be studied include pH, temperature, hydraulic retention time, sludge retention time, organic loading rate, and nutrient concentration.

Additional improvements of microbial communities should be considered such as creating conditions that select for the stable and productive growth of desired microbes, while preventing or limiting growth of organisms that reduce hydrogen yields. Microbial population optimization could be achieved by biostimulation using the additive of various nutrient species specifically for the community, bioaugmentation using the additive of dominant species or efficient hydrogen-producing bacteria into the system, and online process control for maintaining their community.

A successful selection of such organisms, in particular those responsible for hydrogen production, will be used for recovery from off-set reactors by bioaugmentation strategy. To achieve high and stable hydrogen yield and long-term operation, it is necessary to control the growth of undesirable microorganisms such as hydrogen-consuming bacteria, propionic acid bacteria, and lactic acid bacteria via pH adjustment and reducing of H<sub>2</sub> partial pressure. The absence of hydrogen-consuming bacteria leads to relatively high hydrogen concentrations in the biogas and would significantly reduce costs for gas purification. Enhancement of hydrogen-producing bacteria via specific nutrient supplements will improve the reliability and performance of the process. Sludge population optimization strategies under thermophilic conditions shown in **Figure 3**.

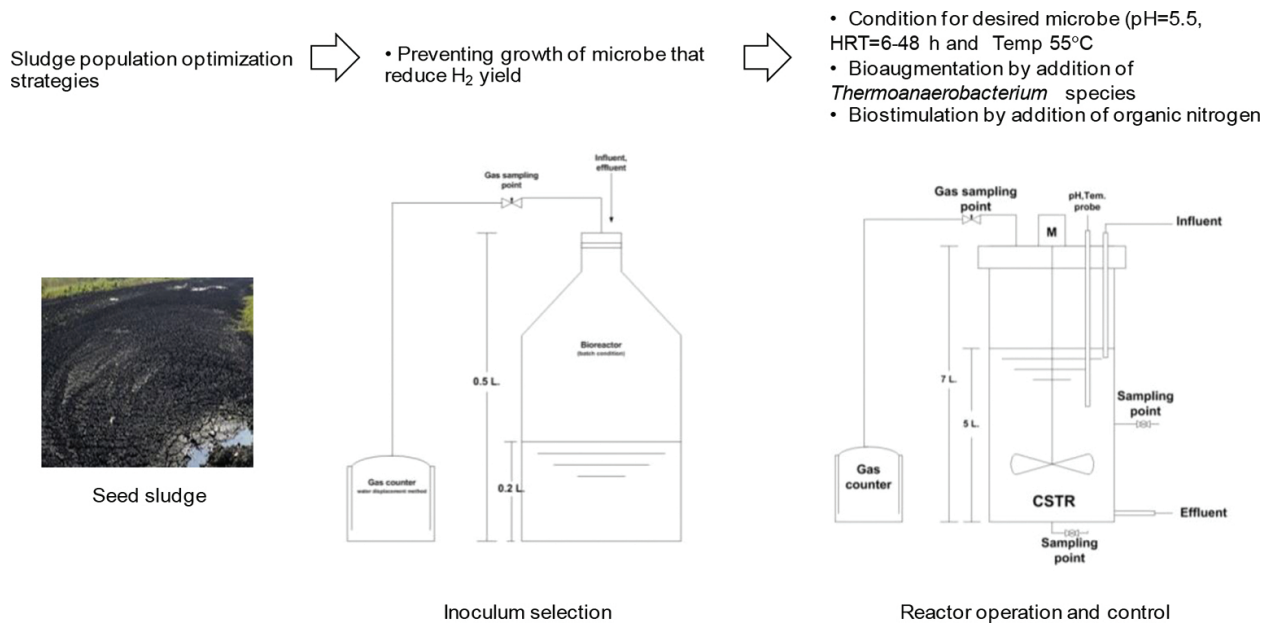


Figure 3. Summary of sludge population optimization strategies under thermophilic conditions.

## 6. Future directions

The use of hydrogen as a clean energy carrier has recently attracted great interest. The cost-effective generation of hydrogen via fermentation will have an important role in this endeavor. Future DHF from organic wastes depends on microbiological community structure and function for enhanced or controllable hydrogen production and reactor. Sludge population optimization aims to obtain the best performance of a system through maximizing the properties of the sludge such as kinetics, yields, and robustness to environmental disturbance. A systematic investigation on the effects of a number of operational conditions on fermentative hydrogen production community and their properties is essential for sludge population optimization. The operational parameters on the appearance of function of microbial species to be studied include pH, temperature, hydraulic retention time, sludge retention time, organic loading rate, and nutrient concentration. Additional improvements on microbial communities should be considered such as creating conditions that select for the stable and productive growth of desired microbes, while preventing or limiting growth of organisms that would reduce hydrogen yields. Microbial population optimization could be managed by biostimulation with the addition of nutrient species specific for their community, bioaugmentation by addition of dominant species or efficient hydrogen-producing bacteria into the system, and online process control for maintaining their community. A successful selection of such organisms, in particular those responsible for hydrogen production, will be useful for the recovery of off-set reactor by bioaugmentation strategy. To achieve high hydrogen yield and long-term operation, it is necessary to control the growth of undesirable microorganisms such as hydrogen-consuming bacteria, propionic acid bacteria, and lactic acid bacteria via pH adjustment and reduction of  $pH_2$ . The absence of hydrogen-consuming bacteria leads to

relatively high hydrogen concentrations in the biogas and would significantly reduce costs for gas purification. Enhancement of hydrogen-producing bacteria via specific nutrient supplements will improve the reliability and performance of the process.

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